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Reduced proximal tubule angiotensin II receptor expression in streptozotocin-induced diabetes mellitus

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Reduced proximal tubule angiotensin II receptor expression in streptozotocin-induced diabetes mellitus. Diabetes mellitus is characterized by alterations in the intrarenal renin-angiotensin system, including decreases in glomerular angiotensin II (Ang II) receptor density. Since Ang II regulates proximal tubule transport function, the present studies examined whether diabetes altered expression of proximal tubule receptors. In basolateral membranes from 14 day streptozotocin-induced diabetic rats, specific binding of ^{125}I Ang II was decreased to 53 ± 8% of control (3.2 ± 0.5 vs. 1.5 ± 0.2 fmol/mg protein; $N = 7$; $P < 0.02$). Similarly, in proximal tubule brush border membranes from diabetic animals, specific binding was decreased to 63 ± 11% of control (1.1 ± 0.2 vs. 0.6 ± 0.1 fmol/mg protein; $N = 9$; $P < 0.05$). Concomitant insulin treatment reversed the decrease in specific binding of ^{125}I Ang II to basolateral membranes (109 ± 26% of control; $N = 3$) and to brush border membranes (85 ± 17% of control; $N = 6$). In order to determine if changes in expression of type-1 Ang II receptors (AT_1R) accompanied the changes in binding, quantitative polymerase chain reaction of AT_1R mRNA was performed and expressed as the ratio of the amplified AT_1R to that of an Msc1/Msc1 internal deletion mutant and normalized to that of β-actin. In total RNA from proximal tubule suspensions of diabetic animals, AT_1R mRNA expression decreased by 38% (21 ± 3 vs. 13 ± 2 cpm AT_1R/cpm deletion mutant/cpm β actin/10^6; $N = 4$; $P < 0.0025$). Insulin treatment reverted AT_1R mRNA expression to control levels (22 ± 3 cpm AT_1R/cpm deletion mutant/cpm β actin/10^6; $P < 0.001$ compared to the untreated group). Since both $AT_{1a}R$ and $AT_{1b}R$ exist in rat kidney, restriction digests of the PCR-amplified products were performed with Hae III, which indicated the presence of both subtypes in rat proximal tubule. Expression of both subtypes decreased in diabetic rats. Therefore, in rats with untreated streptozotocin-induced diabetes, both proximal tubule Ang II binding and AT_1R mRNA levels decrease. Insulin treatment reverses these abnormalities. These findings suggest that, similar to glomeruli, the diabetic milieu leads to decreased expression of proximal Ang II receptors. This defect in receptor expression may contribute to abnormalities in volume regulation and acid/base balance in diabetes.

In the mammalian proximal tubule, angiotensin II (Ang II) is an important regulator of NaCl, $NaHCO_3$ and net volume reabsorption [1]. In addition, Ang II regulates other metabolic functions of this nephron segment and may be a mediator of hypertrophic growth [2]. All of the components of the renin-angiotensin system have been localized to the mammalian proximal tubule [3–5], and it has been suggested that local as well as systemic regulation of the renin-angiotensin system is involved in the mediation of proximal tubule reabsorption.

The actions of Ang II in the proximal tubule are mediated by specific Ang II receptors, present on both apical and basolateral membranes. Recent studies have indicated that in both rat and rabbit, binding of ^{125}I Ang II to either basolateral or brush border membranes is completely inhibited by the type 1 receptor antagonist, losartan, indicating that in proximal tubule, the Ang II receptors are type 1 receptors (AT_1R) [6]. Alterations in Ang II receptor density in the proximal tubule may occur as a physiologic response to changes in intravascular volume [7] and may be involved in pathophysiologic states such as in the prehypertensive spontaneously hypertensive rat [8].

Ang II receptor binding in the glomerulus is decreased in experimentally induced diabetes [9, 10]. The functional significance of this decrease in receptors is not clear, since studies have indicated that administration of losartan to streptozotocin-induced diabetic rats caused decreases in intraglomerular capillary pressure and increases in K_f [11]. In contrast, losartan did not alter proximal tubule reabsorption in diabetic rats [11], although administration of this inhibitor to normal rats produced significant natriuresis and diuresis [12]. These findings suggest that in diabetes, proximal tubules may have decreased responsiveness to Ang II. The present studies were designed to examine whether proximal tubule Ang II receptor density is altered in diabetes. The results indicate that animals with untreated diabetes manifest a significant decrease in expression of AT_1R mRNA, which is associated with decreases in both basolateral and brush border ^{125}I Ang II binding.

Methods

Animals

Diabetes was induced in male Sprague-Dawley rats (200 to 250 g) by a single intravenous injection of streptozotocin (60 mg/kg), as previously described [13]. Only animals that had documented hyperglycemia and glycosuria 24 hours after injection were used. The insulin-treated subgroup received a daily subcutaneous injection of 2 U ultralente insulin/100 g body weight (Eli Lilly, Indianapolis, Indiana, USA) daily, beginning 24 hours after streptozotocin administration. Preliminary studies suggested that this dose provided glycemic control (minimal glycosuria). Animals were sacrificed two weeks after the institution of diabetes. For membrane preparation and RNA extraction, each "*N*" is derived from tissue pooled from three to four animals.

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Renin assays

Plasma and tissue renin activity were measured by radioimmunoassay using previously published methods [11]. Animals were subjected to ether anesthesia prior to collection of blood or organs. For tissue preparation, kidneys were homogenized in 0.1 M Tris-HCl pH 7.4, containing 0.25 mM EDTA and 0.1% Triton X-100, then centrifuged at 10,000 g at 4°C.

Preparation of membranes and isolated nephron segments

Renal cortical membranes were prepared as previously described [13, 14] with protein concentrations measured using the method of Lowry et al [15]. As we have previously described, BLM were enriched in ouabain-sensitive Na^+-K^+ ATPase activity by 7- to 11-fold, while BBM had no significant enrichment in this enzyme. That the relative purification of BLM from the different experimental groups was not different was indicated by demonstration of similar ouabain-sensitive Na^+-K^+ ATPase activity in the BLM preparations [control 29.6 ± 4.8 nmol/mg protein/min ($N = 5$); untreated diabetes 26.9 ± 3.8 nmol/mg protein/min ($N = 5$); insulin-treated diabetic 19.5 ± 4.8 nmol/mg protein/min ($N = 3$)]. BBM exhibited a ~6- to 8-fold increase in alkaline phosphatase activity, while BLM did not have increased activity [6, 13]. Similar relative purification among experimental groups was indicated by similar alkaline phosphatase activity in BBM [control 0.70 ± 0.24 μmol/mg protein/min ($N = 4$); untreated diabetes 0.62 ± 0.21 μmol/mg protein/min ($N = 7$); insulin-treated diabetes 0.52 ± 0.17 μmol/mg protein/min ($N = 5$)]. For preparation of suspensions of proximal tubules, modifications of the method of Vinay et al [16] were used as previously described [14].

Binding to kidney cortical membranes

Binding assays were performed using ^{125}I Ang II as previously described [6]. Briefly, membranes (50 to 150 μg) were incubated at room temperature in a solution of 100 mM NaCl, 5 mM ethylenediaminetetraacetate, 10 mM HEPES (pH 7.5), 100 mM mannitol, 0.5% bovine serum albumin, 0.1 mM $MgSO_4$, 0.5% trypsin inhibitor, 0.005% aprotinin, 0.1 mM phenylmethylsulfonylfluoride and 0.1 nM ^{125}I-labeled Ang II. Under these conditions, equilibrium binding occurred within 10 to 15 minutes for both BLM and BBM preparations. Binding was routinely terminated after 20 minutes by addition of 2 ml of ice-cold stop solution [300 mM NaCl, 10 mM Tris (pH 7.5), 100 mM mannitol, 50 mM $MgCl_2$], followed immediately by rapid filtration through presoaked 0.65 μm filters (DAWP; Millipore Corp., Bedford, Massachusetts, USA) and four washes with 2 ml each of stop solution. Radioactivity bound to the filters was counted in a gamma counter. Specific binding was determined as total binding minus nonspecific binding in the presence of excess unlabeled Ang II (10^{-6} M). For binding curves, the amount of specific binding (total-nonspecific) was determined in the presence of 0.1 nM ^{125}I labeled Ang II and increasing concentrations of unlabeled Ang II.

Quantitative polymerase chain reaction (PCR) of AT_1 receptor

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method [17]. As an internal control for quantitative PCR, a deletion mutant was constructed by cutting rabbit AT_1R clone 3 [6] with MscI (which cuts at two sites in this clone, 304 and 593) and religating. The mutant plasmid was then linearized and used as a template for RNA transcription. For PCR, total RNA (10 μg) and deletion mutant AT_1R RNA (200 pg) were mixed and reverse transcribed using murine reverse transcriptase (Pharmacia, First Strand cDNA Synthesis kit, Piscataway, New Jersey, USA) and a primer specific for the AT_1R. The resultant single strand cDNA mixture was then amplified in a Perkin Elmer GeneAMP 9600 PCR System using Taq polymerase (Perkin Elmer/Cetus). Samples were amplified in the presence of α-^{32}P CTP (New England Nuclear, Boston, Massachusetts, USA; 3000 Ci • mmol; 2 μCi/sample). Preliminary studies determined linearity of response for at least 40 cycles for both intact receptor and deletion fragment, and PCR was routinely carried out for 35 cycles at 95°C for 20 seconds, 55°C for 30 seconds and 72°C for 90 seconds, followed by a 10 minutes extension at 72°C. The employed primers were upstream sense primer, 5′-TGGGAATATT-TGGGAACAGC-3′ and downstream anti-sense primer, 3′-GT-GAATATTTGGTGGGGAAC-5′. Amplification of intact and mutant AT_1R mRNA by these primers yielded 703 bp and 415 bp fragments, respectively. No amplification occurred in the absence of reverse transcription, indicating that genomic DNA was not being amplified. In preliminary experiments, samples of cortex from normal rat cortex and proximal tubule were reverse transcribed and amplified in the presence of the deletion fragment (and in the absence of α-^{32}P CTP), transferred to nylon membranes and hybridized with a cDNA of rabbit AT_1R containing the complete coding region that was labeled by the random priming method to $>10^8$ cpm/μg. Hybrization to the amplified 703 bp and 415 bp fragments was observed. For normalization, parallel samples measured amplification of β-actin cDNA using the primers, 5′-AACCGCGAGAAGATGACCCAGATCAT-GTTT-3′; and 3′-AGCAGCCGTGGCCATCTCTTGCTC-GAAGTC-5′ [18]. Preliminary studies indicated linearity of β-actin mRNA amplification for >40 cycles. Following gel chromatography on 4% agarose gels, the bands corresponding to the PCR products from intact AT_1R, the deletion fragment and β-actin were excised and the radioactivity determined by scintillation spectrometry. Results are represented as the ratio of intact and deletion fragment AT_1R mRNA amplified, normalized to the amount of amplified β-actin mRNA. This method provides a relative comparison of the amount of AT_1R mRNA present among the different experimental groups.

Statistics

Results are presented as the means ± SEM. For statistical analysis, Student's unpaired t-test was employed. For multiple group comparisons, ANOVA and the Bonferroni/Dunn t-test were utilized, with $P < 0.05$ indicating significance.

Results

The rats with streptozotocin-induced diabetes evidenced severe hyperglycemia, as well as glycosuria and mild ketonuria (Table 1). In these rats, there were significant decreases in body weight and increases in the kidney weight/body weight ratio two weeks after induction of diabetes (Table 1). Insulin treatment partially reversed the decreases in body weight, as well as the renal hypertrophy (Table 1). In the diabetic animals not receiving insulin, plasma renin levels were significantly decreased compared to controls [3.9 ± 1.2 ($N = 8$) vs. 8.4 ± 1.0 ng/ml/hr ($N = 6$); $P < 0.05$). Renal tissue renin was not significantly different between control and untreated diabetic animals (control 0.17 ± 0.04 vs. 0.18 ± 0.03 ng/hr/mg protein; $N = 6$). In the insulin-treated

Table 1. Physiologic parameters in streptozotocin-induced diabetes mellitus

	Body weight	Kidney weight	Kidney weight / body weight	Plasma glucose	Urine volume	Urine glucose	Plasma renin	Kidney renin
	g		*%*	*mg/dl*	*ml/day*	*mg/dl*	*ng/ml/hr*	*ng/hr/mg protein*
Control								
x	359	1.36	0.38	90	17	0	8.3	0.17
SEM	9	0.04	0.01	6	1		1.0	0.04
(*N*)	(11)	(11)	(11)	(10)	(11)	(11)	(6)	(6)
Untreated diabetic								
x	229[c]	1.27	0.57[c]	329[c]	86[c]	>500	3.9[a]	0.18
SEM	15	0.08	0.01	17	3		1.2	0.03
(*N*)	(12)	(12)	(12)	(11)	(12)	(12)	(8)	(5)
Insulin-treated diabetic								
X	261[b]	1.13	0.44[a]	158[c]	39[b]	125–250	5.4	0.49
SEM	26	0.09	0.02	11	8		2.0	0.17
(*N*)	(9)	(9)	(9)	(8)	(9)	(9)	(7)	(4)

Results of urine glucose measurements represent semi-quantitative results of urine dipstick analysis. Note that in the untreated diabetic group, all urine glucoses tested >500.

[a] $P < 0.05$ compared to control

[b] $P < 0.01$ compared to control

[c] $P < 0.001$ compared to control

subgroup, plasma renin levels were intermediate between the control and diabetic groups (5.4 ± 2.0 ng/hr/mg; $N = 6$), while renal tissue renin was numerically but not significantly higher than control (0.49 ± 0.17 ng/hr/mg).

To determine whether proximal tubule Ang II receptor density was altered by diabetes, ^{125}I Ang II binding was performed on both basolateral and brush border membranes from control, diabetic and insulin-treated diabetic rats. In basolateral membranes from diabetic rats, specific binding of ^{125}I Ang II was decreased to 53 ± 8% of control (3.2 ± 0.5 vs. 1.5 ± 0.2 fmol/mg protein; $N = 7$; $P < 0.02$; Fig. 1A). The decrease was due to a reduction in the number of binding sites (B_{max} control: 103 fmol/mg protein; diabetic: 35 fmol/mg protein) without alteration in the binding affinity, K_d (4.68 vs. 3.17 nM; Fig. 1B). Similarly, in brush border membranes from untreated diabetic animals specific binding of ^{125}I Ang II decreased to 63 ± 11% of control (1.1 ± 0.2 vs. 0.6 ± 0.1 fmol/mg protein; $N = 9$; $P < 0.05$; Fig. 1C). As with basolateral membranes, there was a decrease in binding sites in the brush border membranes from the untreated diabetics (B_{max} control 32 fmol/mg protein; diabetic 16 fmol/mg protein; K_d control 3.7; diabetic 3.8 nM; Fig. 1D). The AT_1R-specific inhibitor, losartan (10^{-7} M) inhibited specific Ang II binding to both control and diabetic basolateral membranes (percent inhibition: 96 ± 4% and 100 ± 0% respectively; $N = 3$) and brush border membranes (percent inhibition: 94 ± 6% and 99 ± 1%, respectively; $N = 7$). In a subset of animals, insulin treatment reversed the decrease in specific binding of Ang II to basolateral membranes (Fig. 2A; control 2.43 ± 0.52 fmol/mg protein; untreated diabetic 1.47 ± 0.38 fmol/mg protein; insulin-treated diabetic 2.40 ± 0.06 fmol/mg protein; $N = 3$) and brush border membranes (Fig. 2B; control 0.85 ± 0.21 fmol/mg protein; untreated diabetic 0.48 ± 0.06 fmol/mg protein; insulin-treated diabetic 0.65 ± 0.15 fmol/mg protein; $N = 6$).

In preliminary experiments Northern analysis of total RNA suggested decreases in message abundance for AT_1R mRNA in proximal tubule of diabetic rats. To quantify alterations in AT_1R mRNA expression, quantitative PCR was performed (Fig. 3A and Table 2). The results were normalized to amplification of β-actin mRNA, as discussed in the **Methods**. In preliminary experiments, it was determined that the amount of β-actin mRNA present in varying concentrations of total RNA from proximal tubules was not different among the groups. Also, as indicated in Table 2, there were no differences within experiments of β-actin mRNA among the groups. In the untreated diabetic animals, AT_1R mRNA expression was decreased 38% (20.8 ± 1.4 vs. 12.7 ± 1.1 cpm AT_1R/deletion mutant/β-actin/10^6; $N = 4$; $P < 0.0025$). Insulin treatment reverted AT_1R mRNA expression to control levels (23.3 ± 1.8; $N = 4$; $P < 0.001$ compared to the untreated group; Table 2, Fig. 3B).

Rats express two different isoforms of AT_1R, namely $AT_{1a}R$ and $AT_{1b}R$ [19]. These isoforms, which have 96% amino acid identity, are products of two different genes and are differentally expressed in various tissues [19]. mRNA for both $AT_{1a}R$ and $AT_{1b}R$ are found in rat kidney, but no previous studies have determined which subtype(s) is present in proximal tubule. The primers used for PCR in the present experiments amplify both $AT_{1a}R$ and $AT_{1b}R$. In order to determine which subtypes were present on proximal tubule and whether the decrease in AT_1R mRNA represented selective loss of one subtype, PCR was performed on total RNA from proximal tubules of control and diabetic animals and then digested with the restriction endonuclease, Hae III. Hae III digestion of the 703 bp PCR product of $AT_{1a}R$ generates fragments of 194, 112, 110, 95, 69,48, 42 and 33 bp, while digestion of $AT_{1b}R$ generates fragments of 304, 110, 103, 95 and 91 bp. Adrenal expresses predominantly $AT_{1b}R$ mRNA, as shown in Figure 4A, while glomeruli express predominantly $AT_{1a}R$ (data not shown). As can be seen in Figure 4B, proximal tubule contains both $AT_{1a}R$ and $AT_{1b}R$ messages (note that both 304 and 194 bp fragments are detected). In three separate experiments, RNA from proximal tubules of untreated diabetic animals exhibited apparent decreases in message levels of both $AT_{1a}R$ and $AT_{1b}R$ (Fig. 4B). Although these results do not provide quantitative comparison of alterations in $AT_{1a}R$ and $AT_{1b}R$ mRNA expression in diabetes, they suggest that the decreases in ^{125}I Ang II binding are not the result of selective decrease in only one subtype.

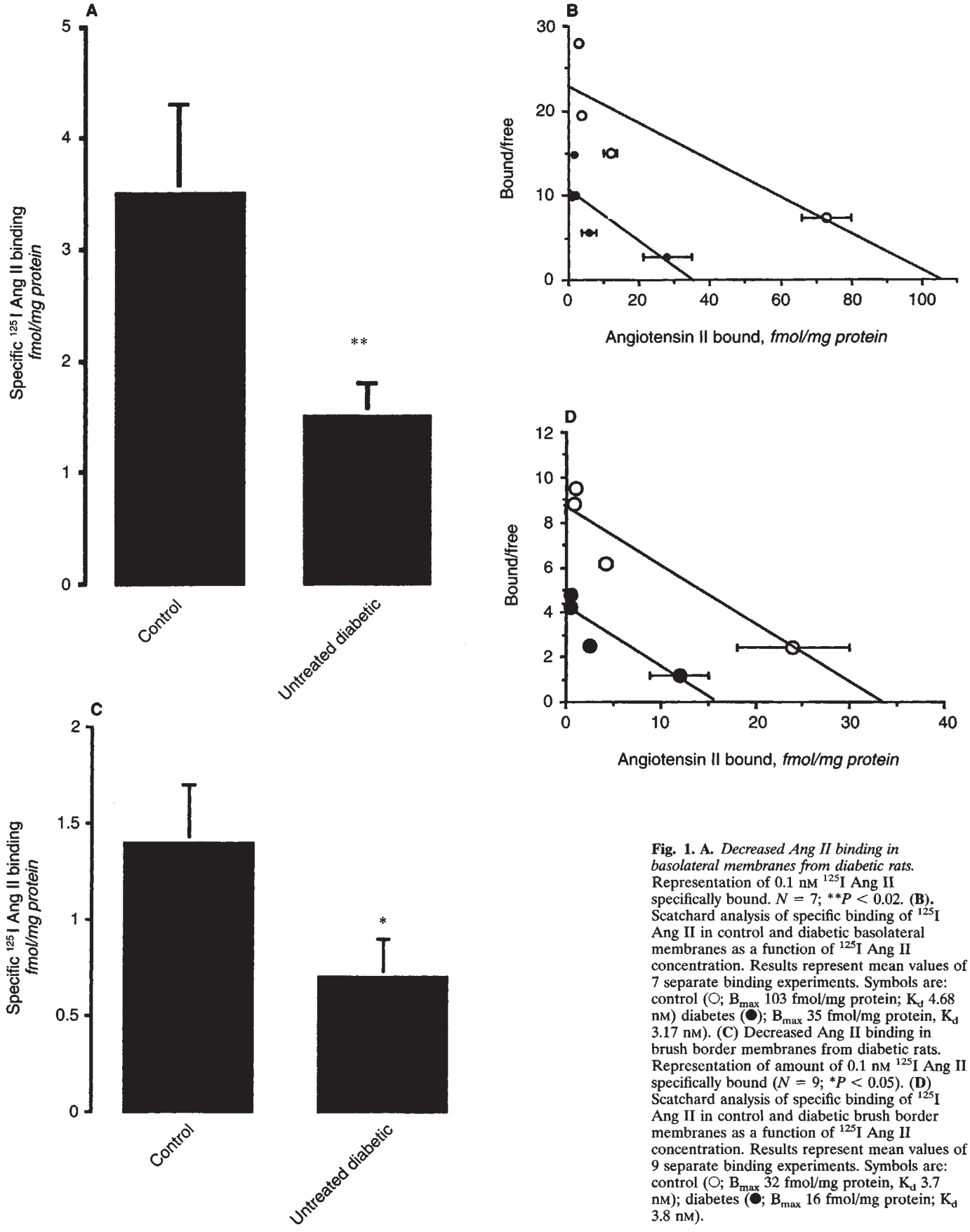

Fig. 1. A. *Decreased Ang II binding in basolateral membranes from diabetic rats.* Representation of 0.1 nM ^{125}I Ang II specifically bound. $N = 7$; $^{**}P < 0.02$. **(B).** Scatchard analysis of specific binding of ^{125}I Ang II in control and diabetic basolateral membranes as a function of ^{125}I Ang II concentration. Results represent mean values of 7 separate binding experiments. Symbols are: control (○; B_{max} 103 fmol/mg protein; K_d 4.68 nM) diabetes (●); B_{max} 35 fmol/mg protein, K_d 3.17 nM). (C) Decreased Ang II binding in brush border membranes from diabetic rats. Representation of amount of 0.1 nM ^{125}I Ang II specifically bound ($N = 9$; $^{*}P < 0.05$). **(D)** Scatchard analysis of specific binding of ^{125}I Ang II in control and diabetic brush border membranes as a function of ^{125}I Ang II concentration. Results represent mean values of 9 separate binding experiments. Symbols are: control (○; B_{max} 32 fmol/mg protein, K_d 3.7 nM); diabetes (●; B_{max} 16 fmol/mg protein; K_d 3.8 nM).

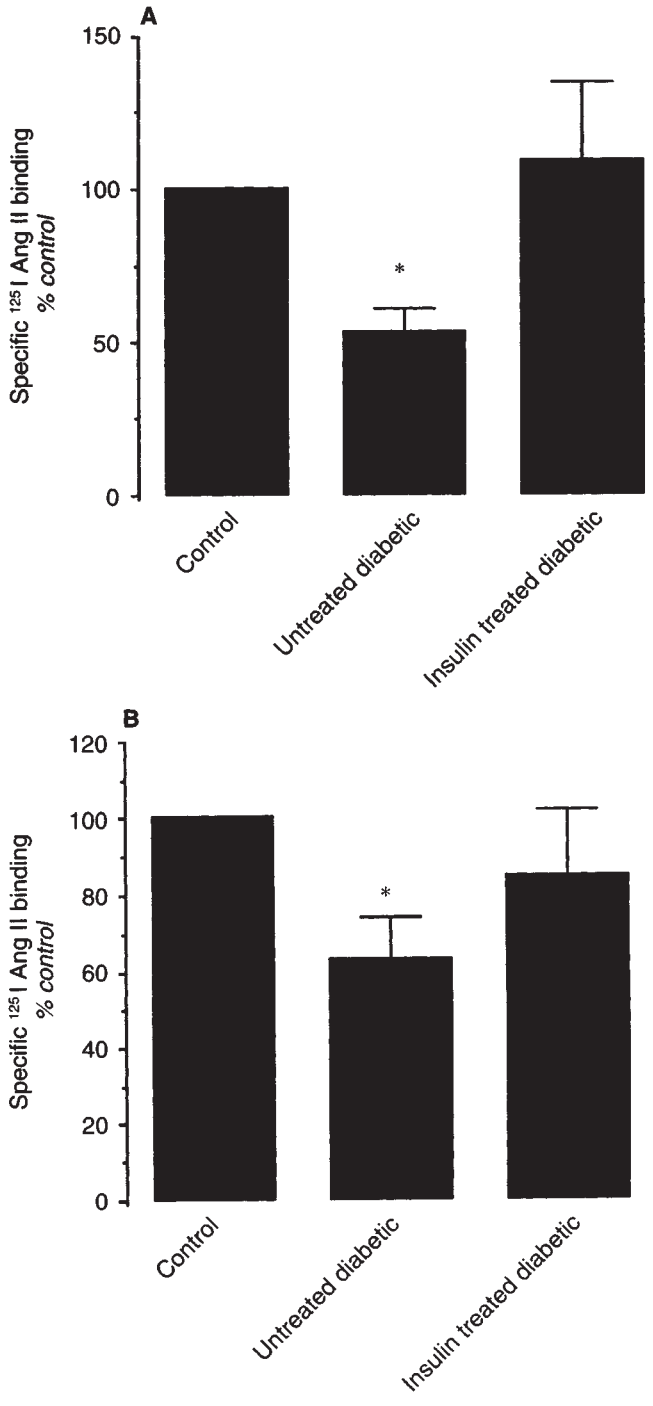

Fig. 2. A. *Insulin treatment reverses decreased basolateral membrane Ang II binding in diabetes.* (*N*) = control: (7); diabetic: (7); insulin treated diabetic: (3). *$P < 0.05$. **(B)** Insulin treatment reverses decreased brush border membrane Ang II binding in diabetes. (*N*) = control: (9); diabetic: (9); insulin treated diabetic: (7); *$P < 0.05$.

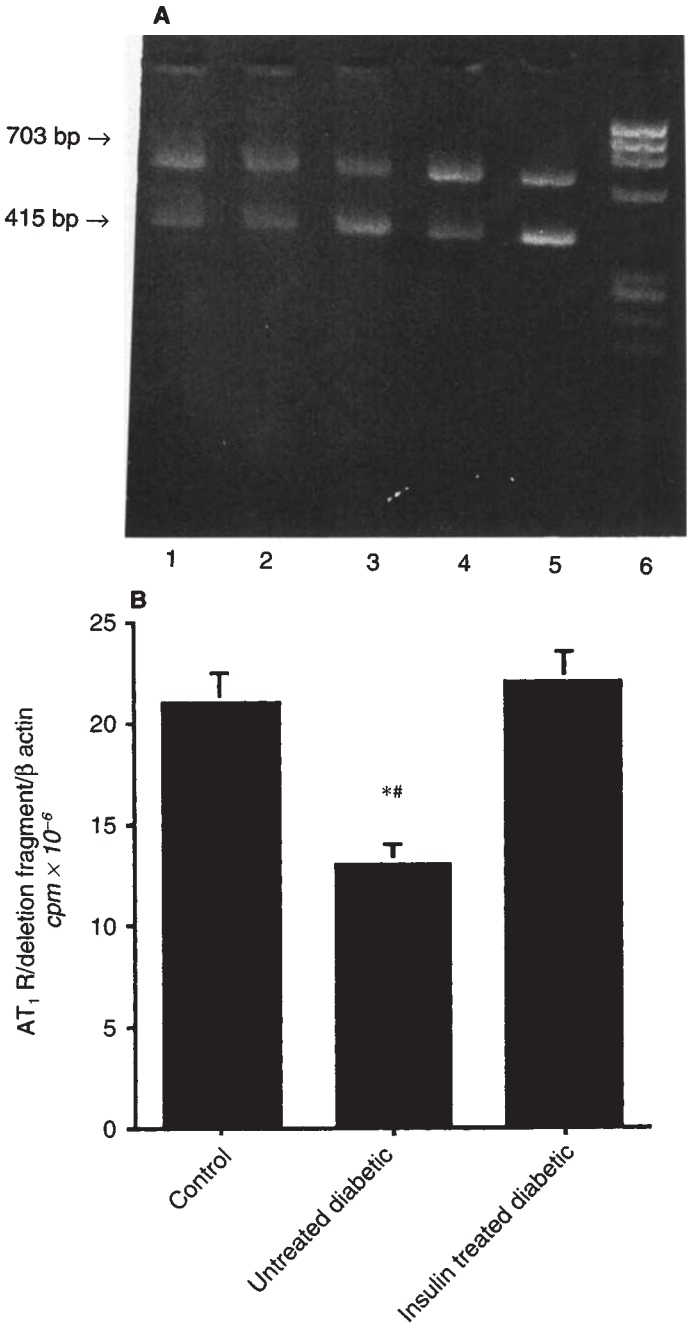

Fig. 3. A. *RT-PCR amplification of AT_1R mRNA in renal cortex and proximal tubules from diabetic rats.* Two hundred picograms of cRNA of an MscI/MscI deletion fragment of rabbit AT_1R was mixed with 10 μg of total RNA from either renal cortex or proximal tubules and reversed transcribed. Following reverse transcription, PCR was carried out for 35 cycles at 95°C for 20 seconds, 55°C for 30 seconds and 72°C for 90 seconds using 5′-TGGGAATATTTGGGAACAGC-3′ as the upstream sense primer and 3′-GTGAATATTTGGTGGGGAAC-5′ as the downstream antisense primer. The amplified fragment of intact AT_1R is 703 bp and the MscI/MscI deletion is 415 bp. lane 1: control cortex; lane 2: untreated diabetic cortex; lane 3: insulin-treated diabetic; lane 4: control proximal tubules; lane 5: untreated diabetic proximal tubules; lane 6: size markers (HAE III Digest of PhiX 174 RF DNA). **(B)** Insulin treatment reverses the decreased AT_1R mRNA expression in proximal tubule in diabetes. Quantitative PCR results from total RNA from proximal tubule suspensions from 4 separate experiments. Results of individual experiments are provided in Table 2 *$P < 0.0025$ compared to control; #$P < 0.001$ compared to insulin treatment.

Discussion

The present studies indicate that untreated diabetic rats had decreased steady state levels of AT_1R mRNA and manifested decreased AT_1 receptor density on both basolateral and brush border membranes. Treatment with insulin reverted both AT_1R

Table 2. Quantitative polymerase chain reaction amplification of AT_1R mRNA from proximal tubule suspensions

	AT_1R/deletion mutant/β-actin/10^6(cpm/cpm/cpm)		
Experiment	Control	Diabetes	Insulin treatment
1	**24.6** (3958/6327/25439)	**12.7** (2662/13979/15019)	**26.3** (4996/9644/19693)
2	**19.8** (2870/3478/41651)	**10.6** (1163/2172/50539)	**19.3** (1977/3020/33859)
3	**20.9** (3591/3468/49546)	**15.7** (1889/2559/46928)	**26.5** (3767/3020/47077)
4	**17.8** (26110/18207/80424)	**11.7** (2724/3239/72003)	**20.9** (29776/19589/72721)
x	**20.8 ± 1.4**	**12.7 ± 1.1**[a]	**23.3 ± 1.8**

Results of quantitative PCR from total RNA from proximal tubule suspensions of four separate experiments. The studies were performed as described in Figure 4. Results in bold represent the ratio of the cpm of ^{32}P CTP incorporated in AT_1R/cpm incorporated into the deletion mutant/cpm incorporated into β-actin/10^6. The cpm of each experiment are provided in parentheses.

[a] $P < 0.0025$ compared to control; $P < 0.001$ compared to insulin treatment

message level and receptor density toward control levels. These studies indicate that that the diabetic state decreased the level of message expression of AT_1R.

The mechanism by which diabetes altered Ang II receptor density in the proximal tubule has not been determined. Wilkes et al have indicated that in glomeruli, untreated diabetes may lead to a generalized decrease in vasoconstrictor receptors [20]. Williams, Tsai and Schrier have reported that in cultured rat aortic smooth muscle cells, both Ang II and vasopression receptors decrease when cells are grown in a high glucose medium [21]. This decrease in receptor density appears to be mediated by protein kinase C, a finding of interest since elevated glomerular protein kinase C levels have been reported in glomeruli from streptozotocin-induced diabetic rats [22]. However, in cultured rat mesangial cells, protein kinase C activation did not appear to be involved in down-regulation of steady state AT_1R mRNA levels [23]. In the present studies, the decreased Ang II binding correlated with an decrease in steady state AT_1R mRNA levels in diabetic tubules. Whether these differences in mRNA levels reflect inhibition of transcription and/or decreased message stability was not addressed in the present studies and will be the subject of further investigation.

In contrast to the present studies, Wolf et al have reported that cultured mouse proximal tubule cells grown in culture medium containing 450 mg/dl glucose have increased ^{125}I Ang II binding compared to cells grown in 100 mg/dl glucose [24]. Therefore, the observed decreases in AT_1R receptor density observed *in vivo* may be the result of other alterations in the diabetic milieu instead of (or in addition to) hyperglycemia *per se*. In this regard, chronic insulin treatment increased ^{125}I Ang II binding in cultured rat mesangial cells [25].

Of interest, in the diabetic animals, plasma renin levels were significantly decreased compared to control animals, and insulin treatment also led to partial correction. Our findings of decreased plasma renin activity in untreated streptozotocin diabetic rats is consistent with previous studies in rats [26–29] and humans [30, 31]. Such studies have indicated that exchangable sodium is increased 10 to 25%, indicating relative volume expansion [28, 30]. In other studies in untreated streptozotocin diabetic rats, renal renin mRNA [26] and tissue renin activity [32] have been reported to be decreased, although in the present studies, no difference in tissue renin activity was noted. When diabetic animals are kept in moderate glycemic control, renal tissue renin activity and mRNA have been reported to be either increased [9] or unchanged [33].

Micropuncture studies have shown that treatment with losartan decreased intraglomerular pressure and increased K_f in poorly controlled diabetes [9]. Indeed, Anderson, Jung and Ingelfinger have suggested that activity of the intrarenal renin-angiotensin system may be increased in such animals, and that there is a specific increase in glomerular angiotensin II production, as suggested by a selective increase in glomerular angiotensin converting enzyme activity [11]. Therefore, it is possible that the recognized decreases in glomerular Ang II binding in untreated streptozotocin-induced diabetes [10, 11] may be secondary to increased local levels of Ang II, since it has been shown that glomerular Ang II binding is decreased in states of increased circulating angiotensin II [34]. Furthermore, in cultured mesangial and smooth muscle cells, angiotensin II administration decreases AT_1R mRNA levels [23, 35].

No previous studies have examined Ang II binding in proximal tubule cells from diabetic animals. However, Anderson et al found that treatment of diabetic rats with losartan did not alter urine volume or electrolyte excretion [11], in contradistinction to the dramatic inhibition of proximal tubule function seen following losartan infusion in normal rats [10]. These studies suggest decreased local proximal tubule angiotensin II production and/or decreased proximal tubule responsiveness to ambient angiotensin II. Of interest, in diabetic rats, proximal tubule angiotensin converting enzyme activity is significantly decreased compared to controls [9].

In other studies from our laboratory, we have recently determined that in rabbit proximal tubules, AT_1R mRNA and Ang II binding decrease in response to captopril treatment and increase in response to a low sodium diet [36]. Similar alterations in Ang II binding have been reported in basolateral membranes from rats [37]. Therefore, decreases in circulating and/or local proximal tubule angiotensin II levels in diabetes may be reflected in decreased AT_1 receptors in the proximal tubule of these animals. However, it is also possible that other alterations in the diabetic state, such as insulin deficiency or corticosteroid excess [38] may contribute to the decreases in Ang II receptors.

In the present studies, the K_d values for Ang II binding are similar to what has been previously described in rat proximal tubule brush border and basolateral membranes [7]. However, the B_{max} values are lower than reported values [7]. The reason for this discrepancy is not apparent; however, since the relative enrichment and marker enzyme activity was similar in all experimental groups, observed differences in relative binding among the groups are meaningful.

The methods used for analysis of AT_1R levels by PCR allow quantitative comparison of expression of message levels among

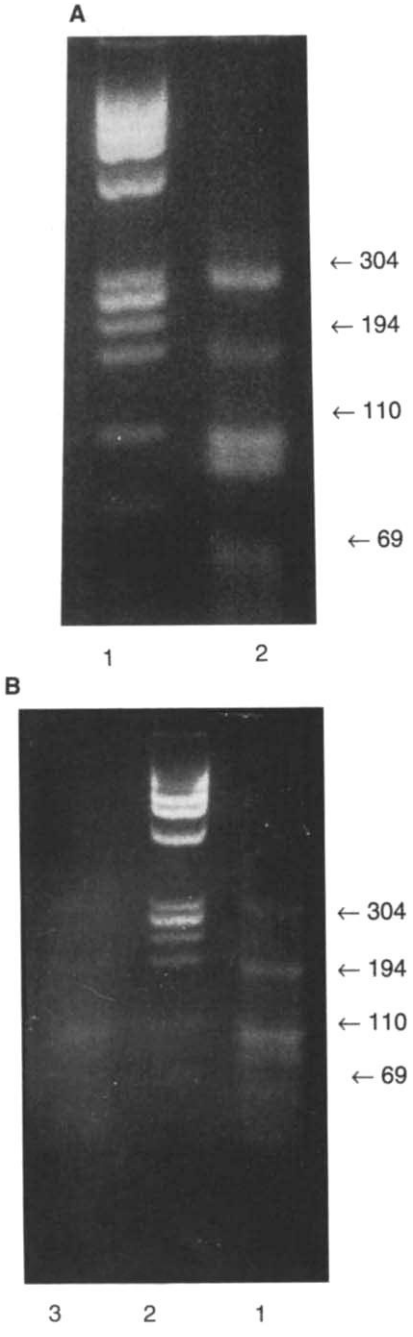

Fig. 4. *Expression of AT_1R subtypes.* Following RT-PCR, the 703 bp fragment was digested with Hae III. Hae III digestion of $AT_{1a}R$ provides fragments of 194, 112, 110, 95, 69, 48, 42 and 33 bp, while digestion of $AT_{1b}R$ provides fragments of 304, 110, 103, 95 and 91 bp. **(A)** Rat adrenal expresses predominatly $AT_{1b}R$. lane 1: size markers (HAE III Digest of PhiX 174 RF DNA); lane 2: control adrenal **(B)** Proximal tubules express both $AT_{1a}R$ and $AT_{1b}R$, and both decrease in untreated diabetes. Lane 1: control; lane 2: size markers; lane 3: untreated diabetes.

experimental groups, although they do not allow quantitation of the actual message concentration. These methods were utilized in order to control for any mRNA degradation occurring during isolation of the proximal tubule or differences in reverse transcription efficiency within experiments. Although it is possible that the deletion fragment of AT_1R mRNA, by virtue of its smaller size, was transcribed and/or amplified more efficiently than the intact AT_1R mRNA, the same amount of deletion fragment mRNA was added to each sample, so that relative differences in the amount of AT_1R that were amplified were not affected.

The physiologic significance of decreased proximal tubule Ang II receptors in diabetes has not yet been determined. Although there is increased proximal reabsorption in the poorly controlled diabetic state, Kumar, Gupta and Spitzer have suggested that this increased reabsorption is due to increased glucose reabsorption occurring with hyperglycemia [39]. In this regard, humans with diabetes mellitus excrete more sodium following institution of sodium restriction than do normal controls because they require longer to reach a new homeostasis between intake and excretion [40]. Whether a defect in proximal reabsorption may manifest itself in the presence of acute volume depletion or angiotensin II infusion must be defined with further studies.

Angiotensin II has also been shown to stimulate proximal tubule ammoniagenesis [41], and it has been suggested that Ang II is an important regulator of proximal tubule acid-base metabolism [42]. It is possible that in association with hyperkalemia, decreased responsiveness to angiotensin II may partially underlie the defect in ammonium secretion that leads to the devolopment of the type IV renal tubular acidosis that is so common in the diabetic population.

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